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ARGININE DESIMIDASE IN *CHLORELLA*

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Abstract—Previous work had shown that *Chlorella vulgaris* rapidly converts arginine to citrulline, suggesting that this organism contains arginine desimidase. An arginine desimidase has been extracted from *Chlorella* and purified 20-fold. Evidence for the desimidase has been obtained by the determination of substrate specificity, identification of the products and the stoichiometry of the reaction. The enzyme requires no cofactors, has a broad pH optimum with a maximum near pH 8·0, has a restricted specificity and is sensitive to thiol reagents.

INTRODUCTION

UNICELLULAR algae such as *Chlorella* and *Scenedesmus* are widely employed for metabolic studies (e.g. photosynthesis) because of their ease of handling and low sampling error. These algae are convenient for investigations of nitrogen metabolism because they readily use not only ammonia and nitrate, but also such organic nitrogen compounds as urea, arginine, citrulline, and ornithine.[1,2] Walker and Myers[3] and Baker and Thompson[2] have reported that *Chlorella* converts arginine to citrulline, and both groups of workers deduced that *Chlorella* must contain arginine desimidase (arginine deiminase = arginine iminohydrolase—E.C.3.5.3.6.). To date this enzyme has been demonstrated only in bacteria[4–8] and yeast.[9]

This paper reports the identification of arginine desimidase in *Chlorella*, and some characteristics of the algal enzyme.

RESULTS

Purification

Arginine desimidase from *Chlorella* has been purified 10- to 20-fold (Table 1). Extensive efforts to obtain further purification by standard procedures have been unsuccessful. This failure has been due partly to the lability of the purified enzyme. For example, a crude extract lost no activity in six days at 0°, whereas a tenfold purified enzyme lost 80 per cent of its activity under the same conditions. However, this increased lability with purification does not entirely explain the failure to purify the enzyme more completely, and a satisfactory explanation has not been obtained.

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TABLE 1. SUMMARY OF ENZYME PURIFICATION

Enzyme fraction	Total volume	Protein	Enzyme activity	Specific activity	Purification	Yield
	(ml)	(mg)	(units)	(units/mg)	(fold)	(%)
Crude extract	87	433	20·9	0·048	1	100
First protamine supernatant	137	397	19·2	0·048	1	92
Ammonium sulfate pellet	8	70	16·9	0·241	5	81
Second protamine pellet						
extract 1	1	4·62	1·35	0·292	6	6
extract 2	3	5·04	4·00	0·795	17	19
extract 3	3	2·46	1·68	0·684	14	8
extract 4	3	1·80	1·70	0·945	20	8
extract 5	3	0·90	0·30	0·333	7	1
extract 6	3	1·20	0·20	0·167	3	1
extract 7	3	0·78	0·22	0·283	6	1

Stoichiometry

The reaction catalyzed by the *Chlorella* enzyme appeared to be a straightforward hydrolysis, as shown in equation 1. Nevertheless, it appeared desirable to determine the stoichiometry of the reaction.

$$\text{Arginine} + H_2O \rightarrow \text{Citrulline} + NH_3 \qquad (1)$$

The ratio of citrulline to arginine was determined by the use of radioactive arginine (cf. Experimental) and that of ammonia to citrulline was determined from colorimetric determinations of these two compounds. Both these ratios are essentially 1:1 (Table 2) and therefore, the equation given appears to be a correct statement of the overall reaction catalyzed by the enzyme.

TABLE 2. STOICHIOMETRIC RATIOS OF ARGININE, CITRULLINE, AND AMMONIA

Substances compared	Ratio
$\frac{\text{Citrulline}}{\text{Arginine}}$	0·912*
$\frac{\text{Ammonia}}{\text{Citrulline}}$	0·981†

* Disintegrations per minute were 17,168 and 18,820.

† Amounts in μmoles were 0·605 and 0·617.

See text for details.

The Effect of the pH *of the Incubation Mixture*

The effect of pH on reaction rate is shown in Fig. 1. Despite the discontinuities imposed by the necessity of using several buffers to cover the desired pH range, it is apparent that the curve has a flat optimum between 7·0 and 8·25.

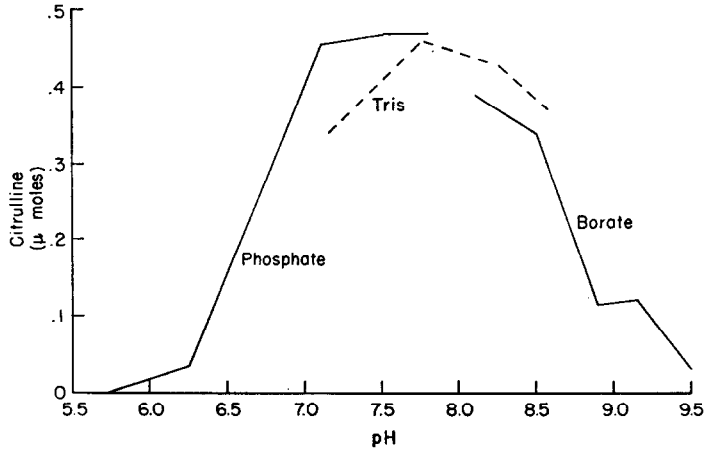

FIG. 1. THE EFFECT OF pH ON ENZYMATIC ACTIVITY.

Assays were carried out under standard conditions, except for the buffer. Each tube having phosphate buffer had 0·5 ml of 0·25 M buffer; the tubes with Tris had 0·5 ml of 0·25 M buffer. Borate buffers were prepared by mixing 0·05 M Na tetraborate with 0·2 M boric acid or 0·2 M NaOH and 1 ml of buffer was added to each tube. The pH values plotted were determined on the complete incubation mixture.

The Effect of Temperature and Time on Stability of the Enzyme

Crude extract has been kept frozen for 42 days with negligible loss of activity. At 37° there is little or no inactivation in 1·5 hr since the amount of citrulline produced is essentially a straight line function of the time of incubation over this period. At higher temperatures, inactivation is rapid, as shown in Table 3.

TABLE 3. EFFECT OF TIME AND TEMPERATURE ON LOSS OF ENZYMATIC ACTIVITY

Temperature	Exposure time	Loss of activity
(°C)	(Minutes)	(%)
50	1	19
	5	34
	10	43
60	1	92
	5	96
	10	96

The Effect of Protein Concentration on Reaction Velocity

When a partially purified enzyme preparation (*ca.* 9 × purified) was used, the amount of citrulline produced was directly proportional to the volume of preparation added over a 20-fold range of concentrations.

The Effect of Arginine Concentration on Reaction Velocity

The increase in reaction velocity with higher substrate concentrations yielded a smooth curve that did not go through the origin. At low arginine concentrations, the reaction rates

were lower than expected from the rates at higher arginine concentrations. When the data were plotted by the Eadie–Hofstee method, a straight line was obtained except at lowest substrate concentrations (lowest reaction rates). This nonlinearity at low arginine concentrations results in a "hook" in the curve (Fig. 2) (see Discussion). A Km value calculated from the straight portion of the curve gave an average value of $0{\cdot}95 \times 10^{-3}$ M (three determinations).

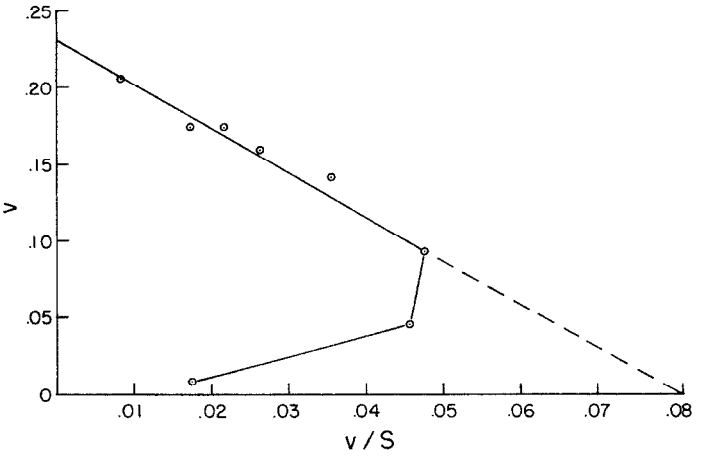

FIG. 2. EADIE–HOFSTEE PLOT OF DATA FROM AN EXPERIMENT IN WHICH THE AMOUNT OF ARGININE WAS VARIED.

Determinations were made under standard conditions. Each point is the average of three determinations.

v is expressed as μmoles of citrulline produced per hour in 3 ml of reaction mixture.
S is the number of μmoles of arginine added to a reaction tube (3 ml).
K_m, calculated from the straight line part of the curve, is $0{\cdot}950 \times 10^{-3}$ M.

Substrate Specificity

Ten compounds closely related to arginine were tested for activity (Table 4), both as substrates for the desimidase and as inhibitors of enzymatic activity towards arginine (see Experimental). Two compounds (arginine methyl ester and arginine amide) had some activity as substrates, whereas alanylarginine and canavanine partially inhibited the activity towards

TABLE 4. SUMMARY OF THE ABILITY OF VARIOUS ARGININE-LIKE COMPOUNDS TO ACT AS SUBSTRATES OR INHIBITORS OF ARGININE DESIMIDASE

Substances not active as substrate or inhibitor	Substances active as substrate	Substances active as inhibitor
δ-Guanidino valeric acid	Arginine methyl ester (6)*	Alanylarginine (57)†
Homoarginine	Arginine amide (31)	Canavanine (28)
Arginylalanine		
Argininic acid		
Agmatine		
D-Arginine		

* Numbers in parentheses indicate percentage of activity as compared with L-arginine.

† Numbers in parentheses indicate percentage of inhibition.

arginine. The enzyme was inactive towards smaller and larger homologs of arginine and was active only when the alpha carbon had the L-configuration. Both an alpha amino group and the carboxyl group were necessary for activity. The enzyme is highly specific, acting only on L-arginine and its ester and amide.

Inhibition

Common enzyme inhibitors at several concentrations were tested for their effect on enzymatic activity (Table 5). The sensitivity of the desimidase for alkylating agents indicated that thiol groups are required for activity. The mild inhibition of the enzyme by cyanide and azide could be interpreted to mean that a metal cofactor is necessary for normal enzymatic activity, but the evidence on this point was inconclusive (see following paragraph).

TABLE 5. EFFECT OF VARIOUS INHIBITORS ON ENZYME ACTIVITY

Compound	Concentration*	Inhibition
	(M)	(%)
pCMB	10^{-6}	12
pCMB	10^{-5}	67
pCMB	10^{-4}	100
pCMB	10^{-3}	100
Iodoacetamide	10^{-3}	31
Iodoacetic acid	10^{-3}	11
Sodium azide	10^{-5}	13
Sodium azide	10^{-4}	32
Sodium azide	10^{-3}	70
Sodium cyanide	10^{-4}	7
Sodium cyanide	10^{-3}	27
EDTA	10^{-3}	7

* All compounds were tested at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. Only those concentrations where an inhibitory effect was observed are included. pCMB is *p*-chloromercuribenzoate. EDTA refers to ethylenediaminetetraacetic acid.

Iron Activation

In some cases the activity of the crude extract was markedly increased by the addition of Fe^{3+} to the reaction mixture. Mn^{2+} has a similar effect but, on a molar basis, it is only about one-tenth as active (Table 6). Because Fe^{3+} has the greater effect, it has been used in preference to Mn^{+2} whenever the addition of a heavy metal seemed desirable.

TABLE 6. THE EFFECT OF IRON AND MANGANESE ON ENZYMATIC ACTIVITY

Conc. of salt in incubation mixture		Citrulline produced	Activation
$FeCl_3$	$MnSO_4$		
(M)	(M)	(μmoles)	(%)
0	0	0·092	—
$2{\cdot}3 \times 10^{-4}$	0	0·310	237
0	$2{\cdot}3 \times 10^{-3}$	0·332	261
$2{\cdot}3 \times 10^{-4}$	$2{\cdot}3 \times 10^{-3}$	0·350	280

The extreme variability of the iron activation (Table 7) is as yet unexplained. It is not due to an effect of the iron on the citrulline determination (see "Citrulline assay"), because an increase in iron above the specified level in citrulline assay resulted in no additional color. This variability may be due in part to variations in the amount of iron in the culture medium. However, dialysis of the enzyme against EDTA did not result in an increase in the amount of activation by iron.

TABLE 7. ACTIVATION OF ARGININE DESMIDASE BY IRON SALTS IN DIFFERENT PREPARATIONS

Experiment number	μmoles citrulline produced*		Activation (%)
	Without iron	With iron†	
64–16	0·092	0·310	237
64–11	0·059	0·157	166
64–17	0·229	0·369	61
64–55	0·116	0·153	32
64–28	0·212	0·244	15
64–50	0·184	0·185	0·5

* Each figure is the average of from two to four replications. The incubation times varied from 30 to 160 min, but were the same within any one experiment.

† $FeCl_3$ concentration was $2{\cdot}3 \times 10^{-4}$ M.

DISCUSSION

The results demonstrate that *Chlorella* contains an active arginine desimidase from evidence on the nature of the substrates and products, the stoichiometry of the reaction and the absence of cofactor requirement. The enzyme can be extracted readily and is soluble rather than particulate since, when the crude extract was centrifuged for one hour at $100{,}000 \times g$, the activity remained in the supernatant.

Since the enzyme can be purified as much as 10- to 20-fold and still retain considerable activity without any of the usual enzymatic cofactors (for comment on the effect of ferric ions see below), it seems probable that no cofactors are essential. The absence of cofactor requirement is characteristic of hydrolytic enzymes.

The erratic nature of the iron activation is not explained. However, it seems unlikely that the metal is acting as a true cofactor for the following reasons: (1) more iron is required than should be needed if it were a typical cofactor, (2) the effect is variable (Table 7), and (3) dialysis against EDTA has failed to increase activation by the addition of $FeCl_3$.

The unusual shape of the Eadie–Hofstee curve (Fig. 2) needs to be interpreted. A possible explanation has come from Webb,[10] who shows a family of curves similar to the one in Fig. 2. In explanation he says: "The only unique situation is where the *back reaction* (italics ours) is occurring and the deviation from the kinetics will depend on the overall equilibrium constant." This situation arises when the product combines with the enzyme. Presumably, the citrulline produced interferes with enzymatic activity, but is readily displaced by arginine at high substrate concentrations. Since arginine contains more energy than citrulline (*ca.* 8·6 kcal., according to Schuegraf *et al.*[11]), it is hard to imagine that a simple hydrolysis of arginine could

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be readily reversible. If Webb's explanation is, indeed, pertinent to the data of Fig. 2, and the other comparable experiments, this might be a hint that the arginine desimidase reaction is actually a two-step reaction, with the first step reversible, rather than the one-step hydrolysis as it appears to be. An alternate explanation for the unusual Eadie–Hoftsee curves is that the arginine may act as an allosteric activator[12] and that this effect takes place at low concentrations.

This is the first definitive evidence for arginine desimidase in organisms other than bacteria and yeast, although there had been presumptive evidence for it in *Chlorella*.[2,3] It can be assumed that the presence of the enzyme in *Chlorella* is related to its lack of arginase.[13,14] Presumably, the desimidase provides the first step for an alternative pathway for the degradation of arginine arising from protein breakdown or excessive synthesis. The presence of the desimidase in *Chlorella* explains the ready utilization of arginine as a nitrogen source by this organism.[1]

The optimum pH of the *Chlorella* enzyme was higher (pH 8·0 vs. 6·8) than the *S. faecalis* enzyme. The enzymes from these two sources were similar in (1) the absence of a requirement for cofactors, and (2) inhibition by canavanine.

No evidence for citrulline metabolism in these extracts was obtained under the standard test conditions, although it is reasonable to expect that a citrulline-degrading enzyme is present in *Chlorella*.

EXPERIMENTAL

Growth of Chlorella

Chlorella vulgaris, var. *viridis*, Chodat, grown in a pure culture on a shaker under constant light, has been used throughout this project as the source of arginine desimidase. All cultures have been grown in the "stock culture medium" of Reisner *et al.*[15], including glucose. Cells have been harvested at any age between 1 and 4 weeks, as convenient, without any evidence for variations in desimadase content.

Citrulline Assay

The citrulline test utilized was a composite of the procedures described by Grisolia[16] and Ratner[17]. It became apparent at an early stage that the addition of ferric and manganous ions greatly increased the reliability of the assay. A modified assay was carried out as follows:

Place the sample in a test tube and adjust the volume to 3 ml with deionized distilled water. Add 4 ml of an acid mixture made by mixing 0·237 g $MnSO_4 \cdot H_2O$ in 398 ml of distilled, deionized water with 1·8 ml 0·1 M $FeCl_3$ solution, 300 ml conc. H_3PO_4, (C.P.) and 100 ml conc. H_2SO_4, technical grade. Mix thoroughly. (Because the acid mixture has a high specific gravity, a Vortex mixer is much more efficient than hand-shaking.) Without delay, add 0·25 ml of 3 per cent diacetyl monoxime in water, shake well, cap with glass, and heat in boiling water in the dark for 15 min. Cool in tap water in the dark for 10 min. Read the absorptivity at 490 nm preferably within 20 min. This assay can be used for as little as 0·05 μmole of citrulline, and amounts up to 0·7 μmole can be determined without dilution.

Ammonia Assay

The Muftic method[18] was utilized for the determination of ammonia, with one slight modification: The phenol-alcohol reagent was made by mixing 25 ml of 88 per cent liquid phenol and 86 ml of absolute ethanol. Muftic's deamination technique was omitted, since the test mixtures contained very small amounts of amino-compounds.

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Protein Assay

Proteins were determined by the Folin-Lowry method, as described by Layne.[19]

Measurement of Enzymatic Activity

The standard incubation mixture contained in micromoles: L-arginine, 25; $FeCl_3$, 0·7; potassium phosphate buffer, pH 8·0, 125; enzymatic preparation; total volume, 3 ml. This was incubated at 37° for 1 hr unless otherwise specified.

Enzymatic activity has been expressed in terms of activity units. One unit is that amount of enzyme responsible for the production of 1 μmole of citrulline in 1 min under standard conditions.

Extraction and Partial Purification of the Enzyme

Throughout the extraction and purification procedures, the temperature was maintained as near 0° as was practical.

1. The crude extract was prepared by a modification of the technique of Shafer *et al.*[20] A summary of the modified technique is as follows: The cells were separated from the medium by centrifugation. Then the cells (2–8 ml) were resuspended in 20 ml of 0·1 M K phosphate buffer (pH = 6·65) and ground in a Virtis[21] homogenizer with 40 ml of glass beads (200 μ dia.) for 20 min at 23,000 rev/min, while maintaining the temperature at 2–5°. After removal of the glass beads by passage through glass wool, the suspension was clarified by centrifugation at 24,500 × *g* for 30 min. The enzyme was present in the supernatant solution which corresponds to the crude extract referred to in this paper.

2. The pH of the crude extract was adjusted to 6·0 by addition of 0·2 N acetic acid. Then enough 1 per cent protamine sulfate solution was added to make a final concentration of 0·23 per cent. After standing for 5–10 min, the mixture was centrifuged for 20 min at 11,000 × *g*. The pale, straw-yellow supernatant was saved and restored to pH 6·65 by addition of 0·2 N NaOH.

3. Enzyme grade $(NH_4)_2SO_4$ was added to give 47 per cent saturation. After standing 5–10 min, the preparation was centrifuged for 20 min at 11,000 × *g*. The precipitate was redissolved in 0·1 M phosphate buffer, pH 6·65, (1 ml for each 10 ml of crude extract). This solution is nearly colorless and is rather stable stored in an ice bath or frozen.

4. The solution was dialyzed, either overnight against 100 volumes of buffer (0·1 M phosphate buffer, pH 6·65, containing 10^{-3} M EDTA solution and $2{\cdot}4 \times 10^{-3}$ M mercaptoethanol), or for 4–6 hr against 30–40 volumes of buffer with two changes of dialysis solution.

5. Protamine sulfate solution was added to the dialyzed solution to make 0·23 per cent protamine sulfate as in step 2. After 5–10 min in an ice bath, the solution was centrifuged 20 min at 24,500 × *g*. The precipitate was extracted repeatedly with 0·1 M phosphate buffer, pH 5·5, containing EDTA and mercaptoethanol as described in paragraph 4. For each 8 ml of solution obtained in paragraph 3, the first extraction was made with 1 ml of buffer, and each subsequent extraction was made with 3 ml. Each time, the pellet was first broken up with a rubber policeman and then stirred with a magnetic bar for from 15 to 45 min. This was followed by centrifugation for 15 min at 24,500 × *g*.

A summary of the purification procedure, with data from a typical experiment, is presented in Table 1.

Stoichiometry

The reaction mixture contained 12·5 μmoles of arginine, with enough radioactive (^{14}C) arginine to produce about 34,000 counts per minute from a 0·25 ml aliquot of the reaction mixture. Enough enzyme extract was added (purified *ca.* 10 ×) to convert about half of the arginine in 2 hr under standard conditions. The mixture contained the usual pH 8·0 buffer, but no $FeCl_3$. Four tubes were set up with active enzyme, and four others with inactive enzyme (heated 3 min in boiling water). Following incubation for 2 hr, all tubes were heated in boiling water for 3 min and then were stored at about 4° until analyzed.

A 0·25 ml aliquot of each tube was mixed with 0·025 ml of 0·25 M KH_2PO_4 and 0·25 ml of water to bring the pH to 7·0 ± 0·10. The mixture was placed on a small column (0·6 × 2·5 cm) of Dowex-50 W-X8 resin in ammonium form to absorb the arginine. When the sample had soaked into the resin, the citrulline was washed through with 4·5 ml of water. The 5 ml of liquid was caught in a scintillation vial, which then contained citrulline plus non-basic radioactive contaminants present in the radioactive arginine. Next, the column was eluted into another scintillation vial with 5 ml of 2 N NH_4OH to remove unreacted arginine which had been retained by the resin. 15 ml of Bray's solution were added to each vial, and the radioactivity was determined by counting the various samples on a liquid scintillation counter, using external standardization to determine the counting efficiency.

[19] E. LAYNE, in *Methods of Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 448, Academic Press, New York (1957).

[20] J. SHAFER, JR., J. E. BAKER and J. F. THOMPSON, *Am. J. Botany* **48**, 896 (1961).

[21] The mention of a specific commercial apparatus and materials does not imply any endorsement by the U.S.D.A., but is used to enable the reader to better understand the procedures.

The difference in counts between the eluate from boiled enzyme tubes and that from active enzyme tubes gave a measure of the amount of arginine converted during the reaction. On the other hand, the difference in counts between the water wash from active enzyme tubes and that from boiled enzyme tubes gave a measure of the citrulline produced. Both differences were automatically "corrected" for any radioactive impurities that might have been in the original arginine sample.

Other 0·25 ml aliquots of the reaction mixtures were used for colorimetric determinations of citrulline and ammonia by the usual methods.

Substrates

Information on substrate activity and inhibition was obtained in a factorial experiment with all possible combinations (six in all) of two substrates: L-arginine, other substrate, and both together; and two enzyme conditions: boiled and active. L-arginine and the other compound being tested were used in equimolar concentrations, usually 12·5 μmoles in a reaction tube. The boiled controls were needed because some of the potential substrates contained a certain amount of ammonia. Two 1-ml aliquots were taken from each tube, one for an ammonia test and the other for a citrulline test. By a suitable comparison of the resulting data, it was possible to correct for ammonia, if any, in the reaction mixture, and to determine whether the added compound was serving as a substrate or acting as an inhibitor.

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